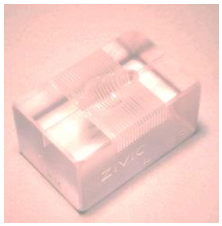
Neurosphere Culturing & Differentiation (Mouse and Human) JLeBelle 2018

(1) Primary tissue culture dissections, dissociation, and culture

Following dissection, human and mouse brain tissue is dissociated into a single cell suspension to generate primary cell cultures. This is done by (1) chopping tissue into small "chunks", (2) treating with a dissociation enzyme for the appropriate amount of time for that enzyme, and (3) mechanical trituration of the enzyme treated tissue. Dissociation enzymes: There are several choices for which dissociation enzyme to use. A method that is not used much anymore is Trypsin dissociation. Tissue treated with Trypsin should be incubated at 37 degrees C for 15 minutes but the enzyme must then be inactivated with soybean Trypsin inhibitor and rinsed. TrypLE is an updated version of Trypsin that is more gentle to cells and does not need to be inactivated, although cells need to be rinsed after treatment. Other enzymes used that are considered more gentle than TrypLE are Accutase, Accumax, and Papain. Papain is considered the most gentle but may also be less effective. Accutase and Accumax are gentle to cells and effective with a short 10 minute incubation at room temperature. They do not need to be inactivated or even rinsed after treatment because the enzyme is inactivated at 37 degrees C (incubator temperature). Accumax is typically used for the dissociation of tissue because it contains DNAse I to break up sticky DNA present from lysed, dead cells to prevent cell clumping. Accutase is primarily used for dissociating healthy cell cultures where little cell death is expected. Single Cell Dissociation: The gold-standard of mechanical trituration is performed with fire-polished glass pipettes (described below) that produces the highest cell viability but may not be strictly necessary for routine passaging of cultures or in cases where large amounts of tissue are available and regular plastic pipette tips can be used.

(2) Primary culture of adult mouse brain



1. Mice given a pentobarbital overdose followed by cervical dislocation to euthanize
2. Brain is removed from the skull and placed into a coronal brain slicing matrix (1 mm sections) and 1-2mm sections are taken through the brain with straight razor blades.

http://www.zivicinstruments.com/brain-slicer-matrix-mouse-adult-1mm-coronal-brainslices-section-x-acrylic.html

3. In the sections which contain the SVZ, tissue punches are taken from both sides of the brain over the SVZ and the tissue are then transferred into labeled tubes containing Hanks Balance Salt Solution (HBSS).

http://shopping.roboz.com/micro-scissors-micro-forceps-groups/Tissue-Punch/HeathCurette-4-1mm-Sharp-Blunt

[Note that if one is very fast at removing the brain and getting the brain sectioned no cooling of the brain is necessary. However, if one is slower at removing the brain it is best to put the brain into cold HBSS and place the brain, cutting matrix, and razor blades in the freezer for 10 minutes. This will prevent "mushy brain" and difficulty cutting.]

Then proceed with this protocol:

☞Centrifuge tissue in HBSS at 1000rpm for 5min
☞Remove media being careful not to disturb the pellet
☞Resuspend in 200ul of Accumax enzyme [thaw Accumax at room temp, do not put in water bath as enzyme degrades at 37 degrees temp of bath or incubator]
☞Break up largest chunks of tissue with pipette right away then leave smaller tissue pieces to incubate at room temperature for 15 minutes
☞Add 600ul of complete media and pipette 8-10 times until most cells are in suspension. [There may be some tissue, especially white matter, that will not break up; do not pipette excessively to break up everything or a large amount of cell death will occur. Using fire-polished (FP) glass pipettes of decreasing diameter increases cell survival while breaking up the tissue. A series of 5 FP pipettes with decreasing diameter opening should be made using the portable Bunsen burner. Do not use the portable Bunsen burner to fire polish the glass pipettes inside the hood. This must be done outside of the hood or you will damage the hood filter system. After smoothing and shrinking the pipette tips opening with the flame, place them (tip up) in an empty pipette box in the sterile hood. Tissue should be gently pipetted up and down 3-4 times with each FP pipette starting with the largest opening down to the smallest. Regular plastic serologic pipettes are OK for triturating most tissue but the fire-polished glass pipettes are best when cell viability is important (i.e., when cell number is low).]
☞Filter cell suspension with a 40-70 uM cell filter
☞Spin cells 1000rpm x 5 min
☞Remove supernatant
☞Re-suspend cell pellet in 1ml complete media
☞Remove 10ul of the cell suspension and combine it with 10 ul trypan blue dye. Then count the cells using either "by hand" using a haemocytometer or using the automated cell counter. [If cell suspension is too dense the 10ul sample of the cell mixture may need to be diluted before mixing with the trypan blue dye. The dilution used will need to be taken into account when calculating the true final density of the cells.]

Re-suspend cells in appropriate conditions for either expansion or for experiment.

(2) Plating neural stem and progenitor cultures (human or mouse) as a monolayer culture

[We typically grow neural cultures as neurospheres but there may be experiments of specific cells that need to be grown as a monolayer instead. To “force” cells or “encourage” them to grow adherent we treat the culture vessel with compounds that attract their strong adherence. First we treat with poly-l-lysine. Polyamino acids facilitate the attachment of cells and proteins to solid surfaces in biological applications. When adsorbed to the culture surface, it increases the number of positively-charged sites available for cell binding. Poly-L-Lysine is a charge enhancer; therefore, it can be used for coating many surfaces. PLL is toxic to cells though so after binding to the flask surface it has to be thoroughly rinsed and fully dried. Then we treat with diluted laminin. Laminin is a major structural multidomain protein of basement membranes and cells are naturally attracted to attach to it. Plates are recommended for monolayer culture rather than flasks because of the coating procedure.]

☞Coat culture vessel with Poly L lysine (PLL) at room temp for at least 1 hour (dilute frozen stock 1:1000). I usually prepare them the day before I need them and leave the plates in the incubator overnight.
☞Wash 3X with autoclaved double distilled water (in the hood already)
☞Air-dry plates/vessel completely with the lid off of the plate inside the sterile hood (lid of plate should be face-down next to the plate). Usually takes a couple of hours. ☞Add diluted mouse laminin (1:50 dilution) into the culture vessel so that it just coats the surface, incubate for at least 2 hours, remove the laminin right before adding the cells in the culture media. Return plate(s) to incubator.

[You want to plate cells so there is around 30-40% confluence so that there is room for them to grow. If they are at too low of a confluence the lack of cell-cell contact with result in very slow growth. You will want to passage them when they reach 80-90% confluence because many cells that aren’t mutated in some way will go into senescence if complete confluence is reached.]

(3) Routine maintenance/expansion of established neural stem and progenitor cultures (both human and mouse)

1. Cells can be grown as either neurospheres or as monolayers. Mouse brain and human fetal brain cell cultures will easily grow as free floating neurospheres in the media below. Induced neural stem and progenitor cultures often grow as both neurospheres and attached cultures, sometimes simultaneously and sometimes switching in adherence preference between passages. If the cells spontaneously attach and grow as a monolayer no special treatment of the culture vessel is needed. However, cells can be “forced” to grow as an attached monolayer by providing an attractive surface in the vessel. Typical treatments include poly-l-lysine + laminin or poly-ornithine + fibronectin.

2.Cells attached with one of these treatments will need to be lifted off and dissociated using TrypLE enzyme on the cells, otherwise neurospheres are either dissociated using Accumax or chopped. Human cells prefer being chopped for regular passaging if they are

growing as neurospheres. Human fetal neurospheres will senesce and stop growing if passaged with a full dissociation for more than 4-5 passages in a row. Full dissociation is fine for plating cells at a specific density, just not multiple passages in a row. Cells that spontaneously attach to culture vessels and grow as a monolayer are not typically strongly attached and can therefore be "lifted" off the plastic with gentle pipetting of media. However, they will then lift up in sheets that can be collected in a tube, spun down, and then dissociated using Accumax.

High Density – used for expanding the number of cells
10K cells/ml (this density is for a once per week passage for fast-growing cells) or 50K cells/ml for slower growing cells or for faster expansion of cells (twice per week passage for fast-growing cells)

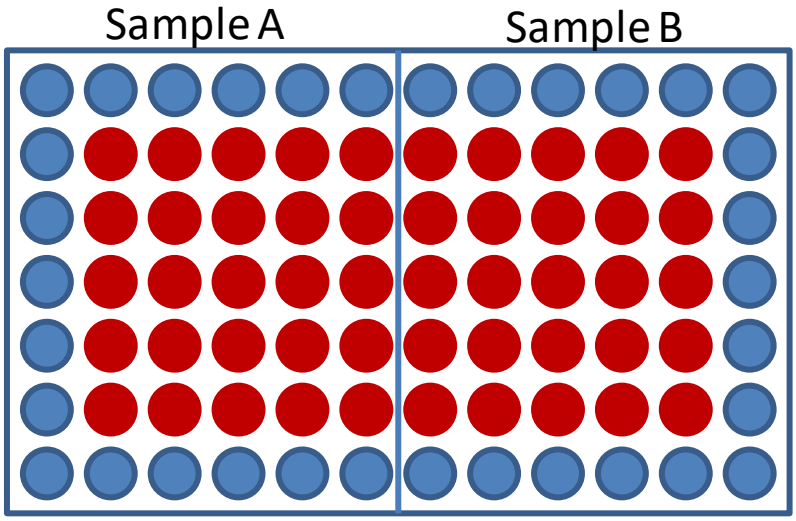
Complete Media:	Volume added	Final Concentration
DMEM/F12	48.5 ml	1X
B27	1 ml	1X
Pen-Strep	500ul	100U/ml
Heparin	50ul	5ug/ml
Basic FGF	40ul	20ng/ml
EGF	25ul	50ng/ml

Feed cultures every 3-5 days with growth factor; if media turns yellow, feed cells with new media as well (75% media change). [The B27 is the serum replacement supplement; the Pen-Strep is antibiotic to prevent infections; heparin is used to stabilize the FGF and EGF and prevents their rapid breakdown at incubator temperatures; both FGF and EGF are required for cultures from adult brain but embryonic cultures do not require EGF]

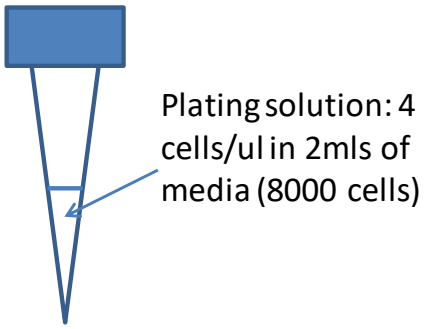
Clonal Density – used for assessing stem cell self-renewal
Clonal Density is 100 cells/ml for most cells.

[Because there is not definitive marker for neural stem cells, the only way to assess the number and self-renewal of stem cells in a culture or in a particular condition/group in and experiment is using a functional assay called the serial clonal neurosphere forming assay. It is not enough to grow neurospheres at clonal density once. In order to assess the presence of neural stem cells you must be able to demonstrate that there is self-renewal. To do this the neurospheres must be passaged at clonal density 3-4 passages consecutively. In addition, you have to also differentiate the spheres to show that the clonal spheres are multipotent at every serial passage.]

Using a high volume multi-channel pipette & a media "boat" add 195ul of complete media to the inner wells of the plate [Use PBS in the outer wells of the plate to prevent media evaporation]. Then the cells are seeded into the wells in 5ul of media [the final density in the wells should be 100 cells/ml (0.1 cells/ul) so you put **20 cells in 5ul into each well** making the final volume 200ul of media per well].



To do this make a clonal plating solution at a density of **4 cells/ul** and then pipette 5 ul per well [=20 cells]. Ideally, make this up in at least 2 mls to use a multi-channel pipette and media boat [2ml =8000 cells]. This will be a much larger volume than you need to add to the plate but it is enough to easily pipette from the media boat. [Always vortex/mix cell suspension right before making up the clonal plating solution and before putting that solution into the media boat for plating].



In addition to the clonal plate used for counting sphere number and diameter, a "sister culture" at clonal density in a T25 (small) flask is made up at the same time [100 cells/ml in 10 ml = 1000 cells per flask]. This sister clonal flask will be used for the next serial clonal passage. The clonal density assay of self-renewal requires 3-4 serial clonal passages to establish self-renewal. Clonal spheres for differentiation to establish multipotency can also be taken from the sister clonal flasks.

Feed cultures with growth factor every 3-4 days
Mouse cells are typically ready for counting 5-7 days after plating at clonal density but GBM cells typically take longer (2-3 weeks). [Waiting too long before counting with fast dividing cultures like the mouse cells can result in neurospheres "merging" which invalidates the assay.]

(4) Passaging

[Spheres must be passaged frequently because as the spheres grow their diameter increases and this prevents the cells at the center of the sphere from having access to the culture media after they grow past a certain size. The deprived cells at the center of the

sphere die. Spheres that appear bright throughout and are less than 200 uM are healthy but larger spheres have a necrotic core that appears dark in the center when looking at them under the microscope. Therefore, to minimize cell death and to maximize proliferation mouse spheres should be broken up into a single cell suspension frequently (at least once per week for fast growing mouse cells). [Human fetal neurospheres, on the other hand, need to be passaged by chopping which is a different methodology.]

☞Remove cells and media from culture flask (if some cells have adhered take only what is floating, depending on the experiment)

[If spheres are large enough (visible to naked eye), tip the flask on its side and wait 1-2 minutes for spheres to sink to the bottom. Then collect the bulk of the spheres in a smaller volume of media. This tipping/sinking method can also be used to do a 75% media change when media is turning yellow-ish but you are not ready to passage the spheres as long as the spheres are large enough to sink.]

☞Centrifuge 1000rpm for 5min

☞Remove media being careful not to disturb the pellet [tip tube to the side and suction only near to he very top edge (meniscus) of the media to avoid suctioning or dislodging the cell pellet.]

☞Add 200ul Accumax (thawed in fridge or at room temp) and incubate 15 min at room temp

☞Add 400 ul complete media

☞Triturate with fire polished glass pipettes of descending diameters or sterile plastic serologic pipette

☞Add 400ul of complete media for a total volume of 1 ml (vortex/mix thoroughly)

☞Remove 10ul of cell mixture and combine it with 10ul trypan blue and count as described above

☞Replate the cells at your desired density in new flasks: high density (bulk) cultures = 10,000 to 50,000 cells/ml or clonal density 100 cells/ml. [Note that the Accumax does not need to be rinsed off of the cells because it will break down automatically when the cells are placed back into the incubator at 37□C. Neurosphere cell suspensions also do not need to be filtered (unless you are preparing them for FACS) because the amount of cellular debris from passaging is negligible.]

Human Neurosphere Chopping (Passaging)

Cells grown in Complete Media:

Volume added	Final Concentration	
DMEM/F12	48.5 ml	1X
B27	1 ml	1X
Pen-Strep	500ul	100U/ml
Heparin	50ul	5ug/ml
Basic FGF	40ul	20ng/ml
EGF	25ul	50ng/ml

Feed cultures every 3-5 days with growth factor; if media turns orange or yellow, feed cells with new media as well (75% media change). If neurospheres are easily visible to the naked eye then the media change can be done by tipping the flask on its side and letting the spheres settle down to the bottom corner of the flask, then suction off 75% of the spent media and replace with new. If the spheres are still too small to sink to the bottom (barely visible with the eye) then all the media would need to be removed and spun down in centrifuge, supernatant removed, and then the cell pellet put back into the flask with new media. It would be very unusual for the media to become orange/yellow while the spheres were that small. [The B27 is the serum replacement supplement; the Pen-Strep is antibiotic to prevent infections; heparin is used to stabilize the FGF and EGF and prevents their rapid breakdown at incubator temperatures; both FGF and EGF are required for cultures from adult brain but embryonic cultures do not require EGF]

Chopping-Passaging

[Spheres must be passaged frequently because as the spheres grow their diameter increases and this prevents the cells at the center of the sphere from having access to the culture media after they grow past a certain size. The deprived cells at the center of the sphere die. Spheres that appear bright throughout and are less than 200 uM are healthy but larger spheres have a necrotic core that appears dark in the center when looking at them under the microscope. Therefore, to minimize cell death and to maximize proliferation mouse spheres should be broken up into smaller cell aggregates frequently (at least once per week for fast growing cells). Human fetal neurospheres, for example, need to be passaged by chopping rather than enzymatic dissociation into a single cell suspension. Human neural progenitor cultures need cell-cell contact more than murine cultures do and repeated full dissociation to single cells leads to senescence for human cultures, although periodic single cell dissociation does not do this.

☞Tip flask on side and allow spheres to sink to the bottom corner of the flask. Using a "squeezy bulb" and glass pipette, remove the spheres in a minimal amount of culture media and transfer onto the lid of one of the small petri dishes that fits the Mcilwain tissue chopper. [Use the lid of the petri dish because the sides are lower and interfere less with the chopper blade.]

☞Using squeezy bulb and glass pipette, remove remaining culture media from around the spheres on the petri dish cover until as dry as possible.

☞ Make sure chopper is set to 150 uM intervals, then chop spheres, turn petri dish 90 degrees, reset chopper blade back to the same start point and chop again. [Flame chopping blade to sterilize before chopping switch blades between samples to completely avoid risk of transferring cells between cultures.]

☞Add a small amount of fresh media onto the chopped spheres in petri dish, tip dish, and use glass pipette and squeezy bulb to break up the chopped spheres into their

chopped pieces and to “unstick” them from the petri dish. [Do this fairly vigorously to fully dissociate the sphere pieces into their chopped size.]

☞If passaging a flask of cells, the chopped spheres can be split in half, usually replating half of the chopped spheres back into the flask and then either freezing the other half or splitting it into another new flask to scale up the culture.

☞The exact density of replating is not known for this reason but generally splitting a culture in half will allow a steady growth with weekly passaging for most human fetal cell cultures.

(5) Differentiation

Differentiation of intact neurospheres

[Differentiation of intact neurospheres yields 20-30% neurons whereas differentiating the same cells but plated as a monolayer of dissociated cells will yield only approximately 5% neurons. Yields of astrocytes and oligodendrocytes are relatively similar though.]

☞Place sterile glass coverslips into the wells of a 24-well plate [use the glass pipette and the suction to “pick up” the sterile glass coverslips without touch them and drop them into the plate wells. Make sure coverslips all lie flat in the wells.]

☞Coat coverslips with Poly L lysine (PLL) at room temp for at least 1 hour (dilute frozen stock 1:1000). I usually prepare them the day before I need them and leave the plates in the incubator overnight.

☞Wash coverslips 3X with autoclaved double distilled water (in the hood already) ☞Air-dry coverslips completely with the lid off of the plate inside the sterile hood (lid of plate should be face-down next to the plate). Usually takes a couple of hours.

☞Add a 30ul drop of undiluted mouse laminin onto the center of a coverslip, remove the laminin right before placing the sphere(s) onto the coverslip and move the laminin drop onto the next coverslip. [diluted laminin can be used to coat the entire coverslip if plating down cells as a monolayer however it is the undiluted method that is required to ensure that single, intact neurospheres will stick to the glass coverslip effectively]

☞Rinse spheres: remove spheres from flask with as little media/PBS as possible using a glass pipette and “squeezy-bulb” and place into approx 10 mls of growth factor-free media or PBS to rinse any growth factor off of the sphere. Then move the spheres into a second 10 mls “rinse”.

☞Remove individual spheres from rinse media in as small a volume as possible (try to get the spheres into the pipette tip end so when released onto the coverslip the spheres stay within the small 30ul laminin drop area on the coverslip) and place the sphere(s) onto the PLL & laminin coated coverslip very slowly and gently. [Use 1 sphere per coverslip if determining the multipotency of a clonal density sphere. More spheres can be plated per coverslip if cells are being differentiated from non-clonal cultures for other purposes. 10 per coverslip is recommended. Once spheres have been placed on laminin from the very first well in the plate be very, very gentle and careful with the plate making

sure not to bump or jostle it so the spheres do not move off of their coverslip] ☞Put plate (gently!) into the incubator for 30-60 minutes before adding the media to the wells. Spheres will begin to attach so that when you do add the media they don't float off. Don't skimp on this short, attachment incubation.
☞Then very gently and slowly flood each well with 500ul of differentiation (growth factor –free) media by pipetting it down the side of the well. [If you aren't careful the spheres will end up floating off of the glass coverslip and differentiating in the corner of the well on the plastic.] Return plate(s) to incubator.
☞Differentiate mouse neurospheres for 5-7 days (human cells 7-14 days) before fixing and staining.

Differentiation of cells as a monolayer

[Differentiation of intact neurospheres yields 20-30% neurons whereas differentiating the same cells but plated as a monolayer of dissociated cells will yield only approximately 5% neurons. Yields of astrocytes and oligodendrocytes are relatively similar though.]
☞Place sterile glass coverslips into the wells of a 24-well plate [use the glass pipette and the suction to "pick up" the sterile glass coverslips without touch them and drop them into the plate wells. Make sure coverslips all lie flat in the wells.]
☞Coat coverslips with Poly L lysine (PLL) at room temp for at least 1 hour (dilute frozen stock 1:1000). I usually prepare them the day before I need them and leave the plates in the incubator overnight.
☞Wash coverslips 3X with autoclaved double distilled water (in the hood already) ☞Air-dry coverslips completely with the lid off of the plate inside the sterile hood (lid of plate should be face-down next to the plate). Usually takes a couple of hours. ☞Add a 100-200ul of diluted mouse laminin (1:100 dilution) into the well with the coverslip, incubate on the coverslip for at least 2 hours, remove the laminin right before adding the cells in the differentiation media to well. In order to keep the majority of the monolayer of cells on the coverslip rather than growing on the plastic around it, put the desired density of cells on the coverslip in around 80ul of media so that it all sits on top of the coverslip only as long as the meniscus holds it together-so be very gentle one you've pipetted the cell solution onto it. Incubate the monolayer cells for 30 minutes. [typical monolayer density used is around 10K cells per coverslip]
☞Then very gently and slowly flood each well with 500ul of differentiation (growth factor –free) media by pipetting it down the side of the well. [If you aren't careful the cells will end up floating off of the glass coverslip and differentiating in the corner of the well on the plastic.] Return plate(s) to incubator.
☞Differentiate mouse neurospheres for 5-7 days (human cells 7-14 days) before fixing and staining.

Differentiation

Media:	Volume added	Final Concentration
DMEM/F12	45.5 ml	1X
B27	1 ml	1X
Pen-Strep	2.5ml	500U/ml

[Differentiation is achieved solely through growth factor withdrawal leaving the rest of the media formulation unchanged from the growth media. Other factors like retinoic acid are not added, although the B27 we use does contain some amount of RA in it (the exact formulation is proprietary). Vitamin A = retinoic acid; B27 does come in a vitamin-A deficient version if needed. This protocol is used to produce neurons. However, after 7 days in mice and 14 days in the humans the culture would need to be switched to specialized media that is used to maintain/support and promote the maturation of the neurons that have been produced. Without doing this the neurons start to die after the 7/14 day time point.]